

Inhibitory ITAM Signaling by Fc α RI-FcR γ Chain Controls Multiple Activating Responses and Prevents Renal Inflammation¹

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Inhibitory signaling is an emerging function of ITAM-bearing immunoreceptors in the maintenance of homeostasis. Monovalent targeting of the IgA Fc receptor (Fc α RI or CD89) by anti-Fc α RI Fab triggers potent inhibitory ITAM (ITAM_i) signaling through the associated FcR γ chain (Fc α RI-FcR γ ITAM_i) that prevents IgG phagocytosis and IgE-mediated asthma. It is not known whether Fc α RI-FcR γ ITAM_i signaling controls receptors that do not function through an ITAM and whether this inhibition requires Src homology protein 1 phosphatase. We show in this study that Fc α RI-Fc γ ITAM_i signals depend on Src homology protein 1 phosphatase to target multiple non-ITAM-bearing receptors such as chemotactic receptors, cytokine receptors, and TLRs. We found that anti-Fc α RI Fab treatment in vivo reduced kidney inflammation in models of immune-mediated glomerulonephritis and nonimmune obstructive nephropathy by a mechanism that involved decreased inflammatory cell infiltration and fibrosis development. This treatment also prevented ex vivo LPS activation of monocytes from patients with lupus nephritis or vasculitis, as well as receptor activation through serum IgA complexes from IgA nephropathy patients. These findings point to a crucial role of Fc α RI-FcR γ ITAM_i signaling in the control of multiple heterologous or autologous inflammatory responses. They also identify anti-Fc α RI Fab as a new potential therapeutic tool for preventing progression of renal inflammatory diseases. *The Journal of Immunology*, 2008, 180: 2669–2678.

Many kidney diseases progress to end-stage renal disease (ESRD),⁴ and represent a major public health problem worldwide (1). Disease progression is characterized by a persistent inflammatory response that causes irreversible renal glomerulosclerosis and tubulointerstitial fibrosis eventually leading to ESRD. Human nephropathies are frequently associated with leukocyte infiltration, a feature of poor prognosis (2–6). Mice that spontaneously develop lupus-like renal inflammation are protected when they lack FcR γ , the common subunit of

activating Fc receptors on myeloid cells (7). Immune complex glomerulonephritis induced by anti-glomerular basal membrane (GBM) Abs, a disorder that involves leukocyte infiltration, is also largely attenuated in mice lacking activating Fc receptors (8, 9). Likewise, cross-linking of myeloid IgA Fc receptors (Fc α RI or CD89) aggravates IgA nephropathy and anti-GBM nephritis in an FcR γ -dependent manner (10). Macrophages and T cells are important in the inflammation associated with ureteral obstruction, another common cause of ESRD (11–16). Conventional anti-inflammatory therapy for ESRD, based on steroids, immunosuppressants, and angiotensin-converting enzyme inhibitors have limited efficacy on disease progression (17). Treatments aimed at reducing leukocyte infiltration and neutralizing inflammatory chemokines/cytokines represent a new field in immunotherapy, exemplified by the efficacy of anti-TNF- α treatment in rheumatoid arthritis (18).

Inhibitory signaling by ITAM-bearing immune receptors that function as molecular switches between activation and inhibition has emerged as a new homeostatic mechanism with therapeutic implications (19). For example, myeloid Fc α RI, in addition to its proinflammatory functions, can trigger powerful anti-inflammatory effects. The latter are activated by monovalent targeting of Fc α RI by monomeric IgA or by an anti-Fc α RI Fab (clone A77). This inhibitory signaling prevents IgG-induced phagocytosis and is beneficial in IgE-mediated asthma (20). The mechanism involves an inhibitory ITAM (ITAM_i) function of the associated FcR γ adaptor. Similar ITAM_i-mediated inhibition has been described with another ITAM-bearing adaptor, DAP12, in association with triggering receptor expressed on myeloid cell (TREM)-2 or with Siglec-H (21–23).

We have previously shown that monovalent targeting of Fc α RI inhibits responses triggered by coexpressed ITAM-activated receptors

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⁴ Abbreviations used in this paper: ESRD, end-stage renal disease; GBM, glomerular basal membrane; SHP, Src homology protein; TREM, triggering receptor expressed on myeloid cell; ITAM_i, inhibitory ITAM; PEG, polyethylene glycol; siRNA, small interfering RNA; UUO, unilateral ureteral obstruction.

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(20). It remained to be evaluated whether Fc α RI-mediated ITAM, inhibition has a broader action that could target non-ITAM-mediated inflammatory responses. We describe in this study that preincubation with the anti-Fc α RI Fab clone A77 inhibited proinflammatory responses in human monocytes and human monocytic cell lines triggered by various heterologous receptors that use different signaling effectors. The inhibitory mechanism involved activation of Src homology protein (SHP)-1 phosphatase, which neutralizes receptor-activated phosphorylation responses. Moreover, monovalent Fc α RI targeting desensitized autologous responses triggered by IgA immune complexes in human monocytes. We obtained evidence that triggering of the inhibitory pathway was beneficial in experimental models of renal disease and prevented inflammation in both ITAM- and non-ITAM-mediated models. Suppression involved inhibition of renal leukocyte infiltration. These data support the important role of infiltrating leukocytes in kidney disease and suggest that ITAM-mediated inhibition of myeloid cell activation might be beneficial in inflammatory renal disorders.

Materials and Methods

Animals

C57BL/6 and BALB/c mice transgenic for the human Fc α RI (CD89, line 83) and Fc α RI_{R209L} (line 1) were used (10, 24). Genotyping was done by PCR. Mice were bred and maintained at the mouse facilities of IFR02 (Faculté de Médecine, Xavier Bichat, Paris, France). All experiments were done in accordance with national guidelines and were approved by a local ethics committee.

Subjects

Heparinized blood and serum were obtained from 49 patients with biopsy-proven IgA nephropathy and no on-going steroid treatment, and from six healthy individuals. Samples from nine patients with other inflammatory renal diseases (antineutrophil cytoplasmic Abs-associated glomerulonephritis, lupus nephritis) and 10 patients with noninflammatory renal diseases (membranous nephropathy, minimal change, diabetic nephropathy) were also included. A local ethics committee approved this part of the study, and all the patients gave their informed consent.

Cells and cell lines

Human PBMC were isolated from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Enriched (70–80%) monocyte populations were obtained by adherence to plastic as described (10). Mouse peritoneal macrophages were prepared as previously described (10). The RBL-2H3 mast cell transfectants Fc α RI_{R209L}/Fc γ and Fc α RI_{R209L}/Fc γ _{Y268/278F} were maintained as described (20). The human monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C with 5% CO₂ in a humidified incubator. The MonoMac 6 cell line was cultured in the same medium with bovine insulin (9 μ g/ml; Sigma-Aldrich).

Igs and Abs

BALB/c-derived (IgG1) mouse mAbs specific for Fc α RI (clone A77) and the irrelevant control mAb (clone 320.1) were used as Fab fragments (20). Fab was prepared by pepsin digestion followed by reduction with 0.01 M cysteine and alkylation with 0.15 M iodoacetamide at pH 7.5. Complete digestion and purity were controlled by SDS-PAGE. The following Abs were also used: rabbit monoclonal anti-CCR2 mAb (Epitomics), rat anti-type I collagen (Southern Biotechnology Associates), rat anti-Mac1 (Serotec), rabbit anti-phospho-p38 or anti-phospho-ERK MAPK Abs (Cell Signaling Technology), rabbit anti-phospho-JNK and rabbit anti-SHP-1 (Santa Cruz Biotechnology), mouse anti- β -actin mAb (Sigma-Aldrich) and rat anti-mouse CD4 Ab (L3T4; Southern Biotechnology Associates). The developing Abs were rat anti-mouse IgG-biotin, goat anti-rabbit HRP and donkey anti-rabbit HRP, and goat anti-mouse FITC (Southern Biotechnology Associates).

Immune complex preparation

Immune complexes were precipitated from serum by incubation with an equal volume of 3.8% polyethylene glycol (PEG) 6000 (Merck) in PBS (pH 7.4). The precipitate was collected by centrifugation at 3000 \times g for

20 min at 4°C and washed twice with 3.5% PEG; each PEG precipitate was re-dissolved in DMEM (volume equal to the serum starting volume).

Cytokine and chemotaxis assays

Human and rat TNF- α levels were measured by ELISA (R&D Systems). Monocyte chemotaxis was measured in 24-well Micro Chemotaxis Transwell plates (Corning; Costar). THP-1 cells or human PBMC (1.5×10^6 /ml) were placed in the upper chamber, separated from the lower chamber by a polycarbonate membrane (5 μ m pore size). MCP-1 (10 ng/ml in RPMI 1640 medium containing 1 mg/ml BSA; R&D Systems) was added to the lower chamber, and cells were allowed to transmigrate for 2 h at 37°C in humidified air with 5% CO₂. Migrated cells in the lower chamber were counted directly by light microscopy.

Small interfering RNA (siRNA) knock down of SHP-1

Human SHP-1 in MonoMac 6 cells was targeted with a mixture of the following three siRNAs (Eurogentec): siRNA1 (sense strand) CAGGCAC CAUCAUUGUCAU; siRNA2 GAACCGCUACAAGAACAUU; and siRNA3 CAGAGCUGGUGGAGUACUA. A universal scramble-negative siRNA (GCCCCGUCUACAUAUGU) was used as control. MonoMac 6 cells were transfected with siRNA by two successive electroporations 24 h apart. For each transfection, cell density was adjusted to 1×10^7 /ml in electroporation buffer (120 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L KH₂PO₄, 10 mmol/L glucose, 20 mmol/L HEPES (pH 7.0)) before electroporation with annealed siRNAs (0.05 μ M) using an Easyject electroporation apparatus (Eurogentec) at 250 V and 2100 μ F. Two successive transfections were preferred over a single one, to improve the knock down of SHP-1. After each electroporation, cells were cultured in complete medium. Sixteen hours after the second electroporation, cells were incubated with anti-Fc α RI (clone A77) or control Ab (clone 320) for 3 h before stimulation with MCP-1 and TNF- α , respectively, followed by cell lysis. The effectiveness of siRNA treatment was tested by SHP-1 immunoblotting.

Tyrosine phosphorylation assay, immunoprecipitation, and Western blotting

Cells were cultured in 6-well plates at 3×10^6 cells/well in 3 ml overnight at 37°C and were then treated with either PBS or the indicated Fab fragments (10 μ g/ml) for 2 h unless otherwise indicated. Cells were washed in DMEM and stimulated with various agents as indicated. After stimulation, cells were solubilized in lysis buffer (50 mM HEPES (pH 7.4), 0.3% Triton X-100, 50 mM NaF, 50 mM NaCl, 1 mM Na₃VO₄, 30 mM Na₄P₂O₇, 50 U/ml aprotinin, 10 μ g/ml leupeptin), and postnuclear supernatants were prepared. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with rabbit anti-phospho-p38 or anti-phospho-ERK MAPK Abs (Cell Signaling Technology), anti-phospho-JNK (Santa Cruz Biotechnology), anti- β -actin mAb (Sigma-Aldrich) followed by goat anti-rabbit or goat anti-mouse Ig, both coupled to HRP. Filters were developed by ECL (GE Healthcare).

Anti-GBM glomerulonephritis and unilateral ureteral obstruction (UUO) nephritis models

Immune-mediated glomerulonephritis was induced by rabbit anti-GBM in BALB/c Fc α RI transgenic mice (6- to 9-wk-old) using an accelerated model of glomerulonephritis as described (10). Nonimmune mediated nephritis was induced in C57BL/6, Fc α RI transgenic mice, or Fc α RI_{R209L} transgenic mice (10- 12-wk-old) by UUO as described (25). For immunotherapy, animals were treated i.v. with either 100 μ g/20 g body weight of A77 mAb Fab in 200 μ l of PBS or 100 μ g/20 g body weight of 320 mAb Fab in 200 μ l of PBS, for 14 days at 24-h intervals. The first dose was administered 24 h before anti-GBM Ab injection or UUO. On the indicated days, blood samples were collected, animals were sacrificed, and kidneys were processed as described (10). Renal function parameters (proteinuria, serum creatinine, and blood urea nitrogen), and histological and immunohistological parameters were studied as previously described (10). Macrophage infiltration was studied in vivo following injection of Dil-labeled thioglycolate-derived Fc α RI⁺ macrophages obtained from transgenic mice as described (10).

Data analysis was completed by ANOVA for statistical calculation as indicated. Data are reported as mean \pm SD, and values for $p < 0.05$ were considered to represent significant differences.

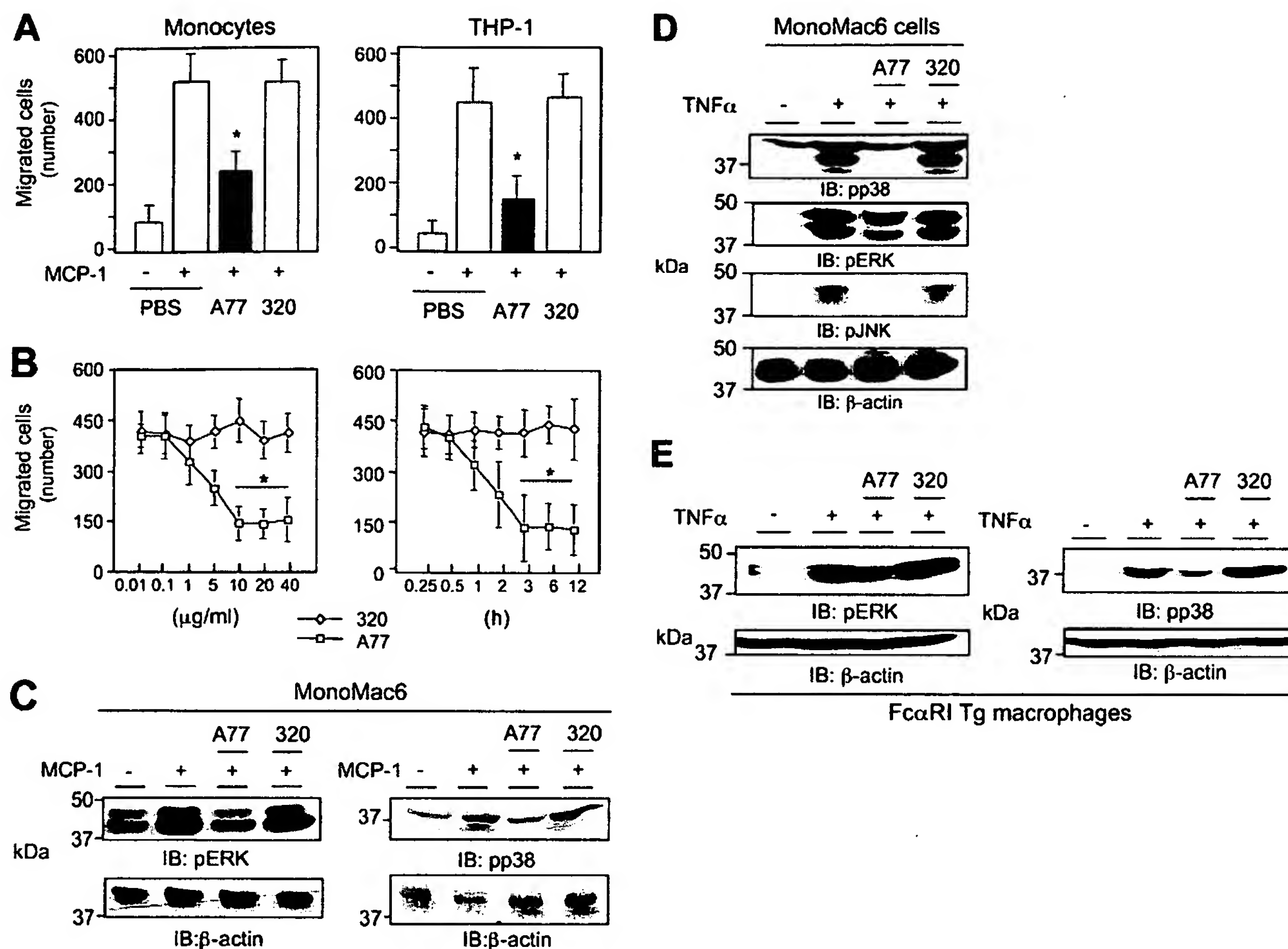


FIGURE 1. MCP-1- and TNF- α -mediated signaling in monocytes/macrophages is inhibited by anti-Fc α RI Fab treatment. **A**, The chemotactic response of human monocytes and the THP-1 monocytic cell line to MCP-1 (10 ng/ml for 4 h) vs medium alone (\square) in the presence of buffer control (PBS) was measured by using 24-well Micro Chemotaxis Transwell plates. MCP-1-mediated chemotaxis was inhibited when cells were preincubated with 10 μ g/ml Fab A77 (\blacksquare) but not after preincubation with 10 μ g/ml Fab 320 (\square). *, $p < 0.05$. **B**, Dose response (left) and kinetics (right) of the effect of Fab A77 vs Fab 320 on MCP-1-induced (10 ng/ml) chemotaxis by human monocytes. **C**, Representative phosphorylation response of p42–44 ERK and p38 MAPKs before and after MCP-1 stimulation (10 ng/ml, 5 min) in untreated MonoMac 6 monocytic cells and after treatment with Fab A77 or Fab 320 (10 μ g/ml) as revealed by immunoblotting with phospho-specific Abs. Reprobing with anti- β -actin is shown as a control for equal loading. **D** and **E**, Representative phosphorylation response of p42–44 ERK, p38 MAPK, and JNK MAPK before and after TNF- α stimulation (50 ng/ml, 5 min) in untreated MonoMac 6 monocytic cells (**D**), Fc α RI⁺ transgenic (Tg) peritoneal macrophages (**E**), and after treatment with Fab A77 or Fab 320, as revealed by immunoblotting with phospho-specific Abs. Reprobing with anti- β -actin is shown as a control for equal loading.

Results

Fc α RI-Fc γ -mediated inhibitory signaling inhibits non-ITAM-activated responses

Fc α RI-Fc γ ITAM_i function can be triggered in the absence of coaggregation (20). We therefore postulated that monovalent targeting in addition to inhibiting coexpressed ITAM-bearing receptors might more generally affect responses of receptors that use different signaling pathways. We analyzed the effect of anti-Fc α RI Fab A77 pretreatment on the chemotactic response to MCP-1 in human monocytes and in THP-1 human monocytic cells that express CCR2, the high-affinity receptor for MCP-1 (data not shown). Fab A77, but not an irrelevant Fab (clone 320), markedly inhibited the MCP-1-induced chemotactic response by both cell types (Fig. 1A). This inhibition was concentration- and time-dependent, with an IC₅₀ around 3 μ g/ml and a maximal effect after 3 h of incubation (Fig. 1B). Key events in MCP-1-mediated chemotaxis, such as p38 and p42–44 ERK MAPK phosphorylation (26), were strongly inhibited in CCR2-expressing MonoMac 6 monocytic cells (Fig. 1C and data not shown). Next, we examined the effect of Fab A77 on TNF- α -initiated activation of p38, ERK

and JNK in MonoMac 6 cells, which can also be activated through TNF receptors (27). TNF- α readily induced phosphorylation of p38, ERK and JNK MAPK (Fig. 1D). All responses were inhibited by preincubation with Fab A77 but not with the irrelevant Fab 320. TNF- α -mediated p38 and ERK phosphorylation responses were also inhibited in peritoneal macrophages isolated from Fc α RI transgenic mice (Fig. 1E), but not in peritoneal macrophages from Fc α RI_{R209L} transgenic mice expressing a Fc γ -less receptor, and hence, ITAM-deficient receptor (data not shown).

The Fc α RI-Fc γ inhibitory mechanism requires SHP-1 phosphatase

Our previous data have shown recruitment of SHP-1 to Fc α RI following monovalent targeting by Fab A77, suggesting that this phosphatase could play a role in inhibitory mechanism (20). To further demonstrate this association, we immunoprecipitated SHP-1 and found that phosphorylated Fc γ is coimmunoprecipitated in activated macrophages following treatment with Fab A77 (Fig. 2A). No association between SHP-1 and Fc γ was found after multivalent cross-linking of Fc α RI (data not

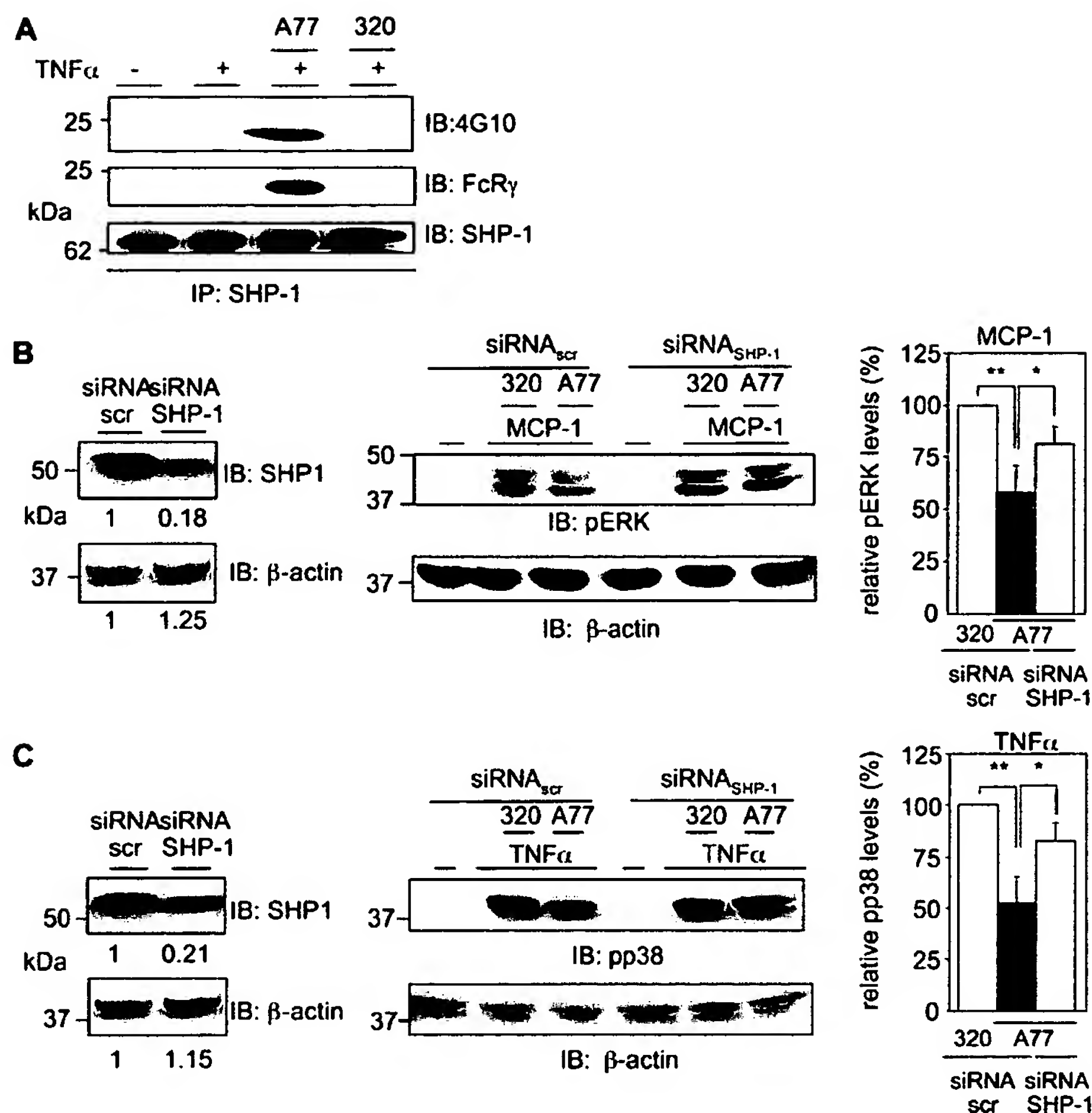


FIGURE 2. Analysis of the role of SHP-1 in the FcαRI inhibitory mechanism. **A**, SHP-1 interacts with phosphorylated FcRγ chain after monomeric targeting of FcαRI. FcαRI⁺ transgenic (Tg) peritoneal macrophages were incubated with buffer, Fab A77, or Fab 320 for 3 h before stimulation with TNF-α (50 ng/ml) as indicated. SHP-1 immunoprecipitates were divided in two samples and immunoblotted with either 4G10 anti-PY goat anti-mouse Ig-HRP or anti-FcRγ plus goat anti-rabbit Ig-HRP. Blots were stripped and reprobed with anti-SHP-1 to judge effective immunoprecipitation. Fab A77-mediated inhibition of MCP-1 (**B**) and TNF-α (**C**) signaling is reversed after specific knock down of SHP-1 phosphatase in MonoMac 6 cells. A representative experiment (*left*) of the expression levels of SHP-1 by immunoblotting with anti-SHP-1 (anti-β-actin as a control for equal loading) after treatment of MonoMac 6 cells with SHP-1 siRNA or scrambled (scr) control siRNA. The relative intensity of the bands is indicated below the blots. A representative immunoblot (*middle*) shows the reversal of Fab A77-mediated inhibition of MCP-1-induced phosphorylation of p42–44 ERK (**B**) and TNF-α-induced phosphorylation of p38 (**C**) after treatment of cells with SHP-1-specific siRNA, but not after treatment with scramble (scr) control siRNA. Anti-β-actin was used as a control for equal loading. The relative levels (*right*) of ERK and p38 phosphorylation in, respectively, MCP-1-stimulated (**B**) and TNF-α-stimulated (**C**) cells preincubated with Fab A77 and control Fab 320 (arbitrarily set to 100%) after treatment with scrambled (scr) siRNA, compared with Fab A77-preincubated cells after treatment with SHP-1 siRNA ($n = 4$). *, $p < 0.05$; **, $p < 0.02$.

shown), confirming data previously described for FcαRI pull downs (20). We therefore directly tested the role of this phosphatase using siRNA knock down in MCP-1- or TNF-α-stimulated MonoMac 6 cells. We focused on ERK activation for MCP-1-induced signaling and on p38 for TNF-α-induced signaling. Both kinases have been implicated in signal transduction leading to inflammatory responses through these cytokines/chemokines (28, 29). As shown in Fig. 2A, siRNA inhibited expression of SHP-1 by >80% in MonoMac 6 cells. Moreover, SHP-1 knock down significantly reversed Fab A77 inhibition of MCP-1-induced responses (Fig. 2B) and of TNF-α-mediated responses (Fig. 2C), supporting SHP-1 involvement in ITAM-mediated inhibition of different receptor systems.

FcαRI monovalent targeting diminishes inflammation in both immunological and nonimmunological models of kidney disease

Our previous study (20) and these findings demonstrate that monovalent targeting of FcαRI broadly inhibits responses that

involve ITAM- and non-ITAM bearing receptors. To determine whether this might have therapeutic implications for renal inflammatory diseases, we analyzed the effect of Fab A77 treatment in immunological and nonimmunological mouse models of kidney disease.

In a first set of experiments, FcαRI transgenic mice were injected with anti-GBM Abs (30) to induce an IgG immune complex glomerulonephritis. Anti-GBM glomerulonephritis involves activation of inflammatory responses by myeloid cells expressing activating IgG Fc receptors (31) and multiple chemokines/cytokines, including TNF-α (32). Mice treated with PBS or irrelevant Fab 320 developed elevated proteinuria, as well as blood urea nitrogen, and creatinine levels (Fig. 3, A and B). All signs of renal disease were significantly attenuated in mice treated with Fab A77. Histological analysis of Fab A77-treated animals also revealed markedly less glomerular expansion and hypercellular changes such as crescent formation associated with aneurysm, sclerosis, and necrosis (Fig. 3C). Renal injury was more severe in mice treated with

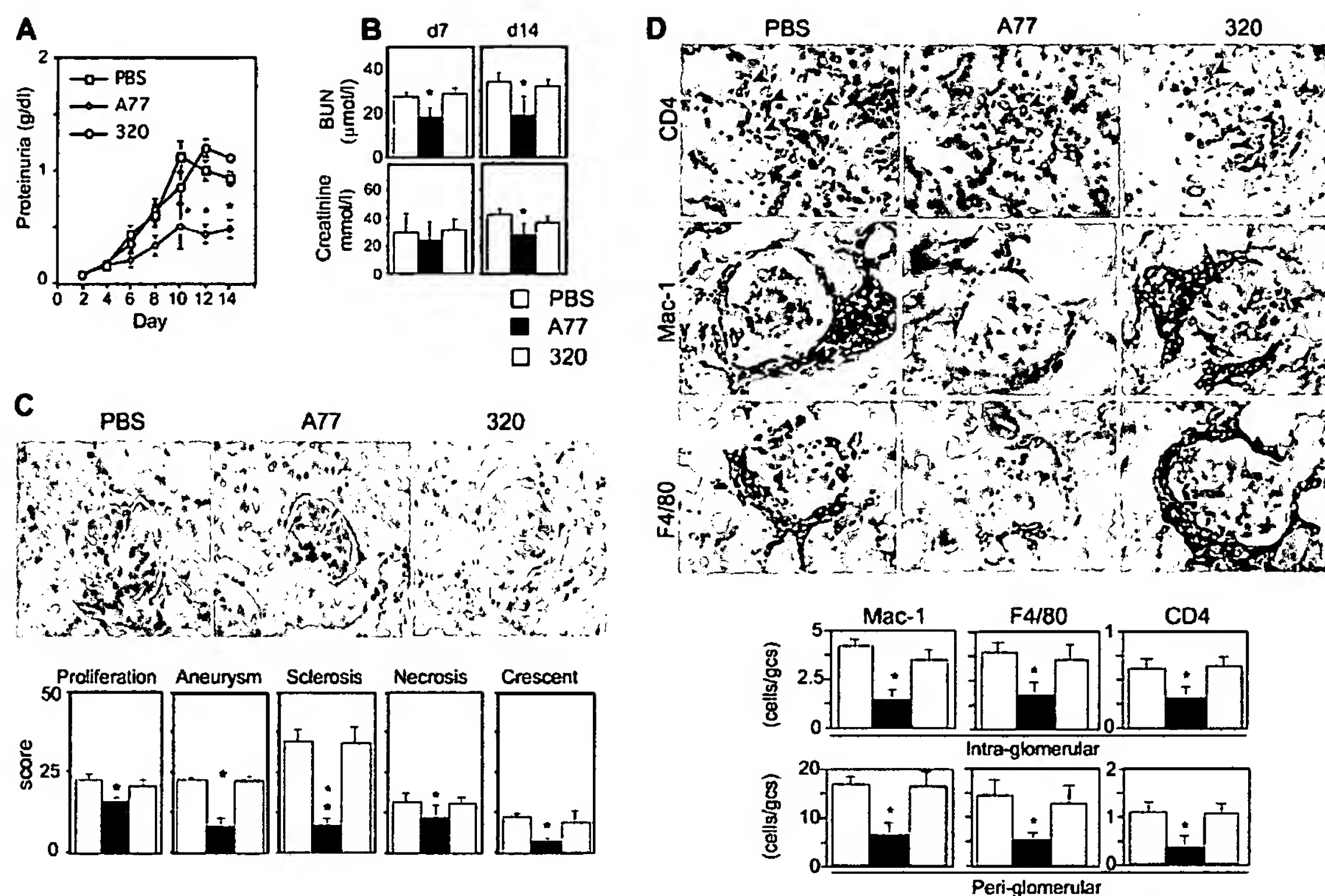


FIGURE 3. Treatment of Fc α RI transgenic mice with anti-Fc α RI Fab A77 improves renal disease parameters in anti-GBM-induced glomerulonephritis. **A**, Evaluation of proteinuria over 14 days in mice after induction of anti-GBM glomerulonephritis and treatment with PBS, Fab A77, or Fab 320 as described in *Materials and Methods*. **B**, Evaluation of blood urea nitrogen (BUN) and creatinine levels 7 and 14 days after induction of anti-GBM glomerulonephritis in mice treated with PBS, Fab A77, or Fab 320. **C**, Histologic analysis (PAS staining) after induction of anti-GBM glomerulonephritis and following treatment with PBS, Fab A77, or Fab 320. Corresponding quantitative evaluation (*below*) of histologic disease parameters by disease severity score is shown. *, $p < 0.05$. **D**, Immunohistological analysis of CD4-, Mac-1-, and F4/80-positive cells following treatment with PBS, Fab A77, or Fab 320, and corresponding quantitative analysis (*below*) of intraglomerular and periglomerular infiltrating cells per glomerular cross-sections (gcs) is shown below the staining. *, $p < 0.05$.

PBS or Fab 320. Fab A77-treated animals had significantly fewer CD11b⁺/F4/80⁺ macrophages and CD4⁺ T cells in glomeruli and interstitial tissue (Fig. 3D), but anti-GBM deposition was similar

in glomeruli of the three groups of mice (data not shown). Thus, Fab A77 treatment showed remarkable efficacy in IgG immune complex-mediated glomerulonephritis.

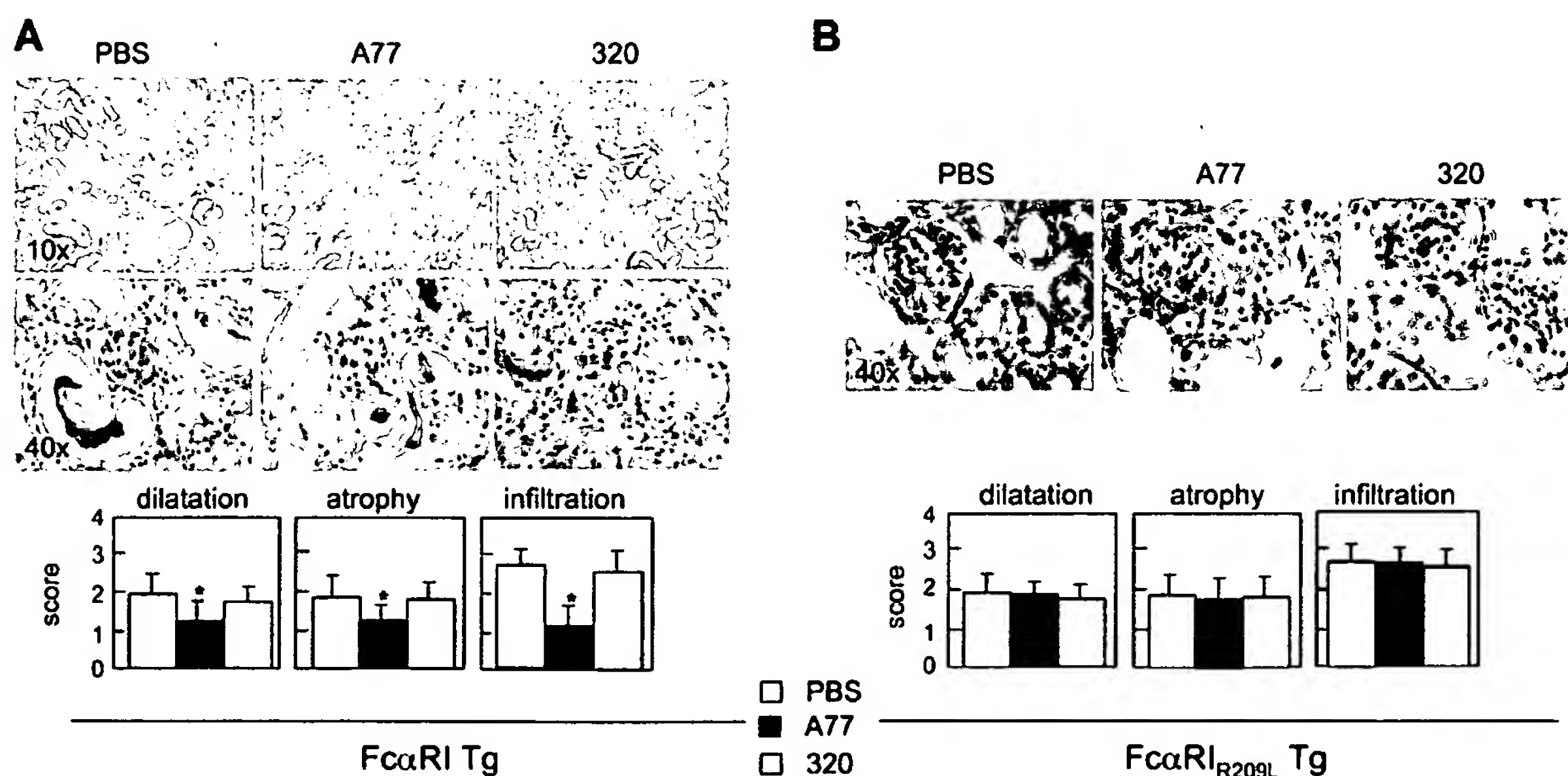


FIGURE 4. Treatment of Fc α RI transgenic (Tg) mice with anti-Fc α RI Fab A77 inhibits renal inflammation after UUO. Histological analysis on day 14 of UUO left kidney sections (PAS staining) from Fc α RI Tg mice (**A**) and from Fc α RI_{R209L} Tg mice (**B**) treated with PBS, Fab A77, or Fab 320. The corresponding quantitative evaluation of histological disease parameters for both analyses is also shown (*below*) by disease severity score. *, $p < 0.05$.

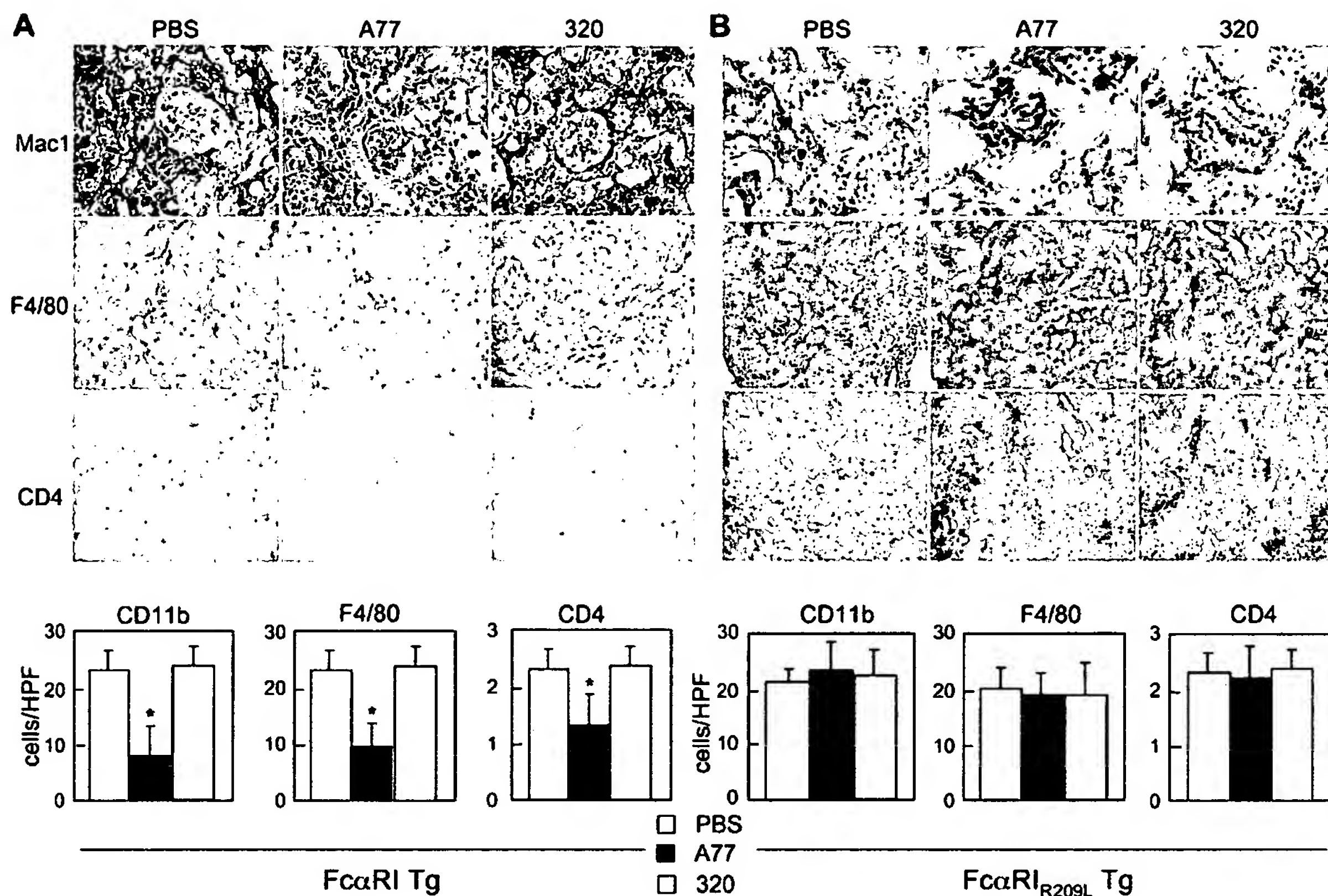
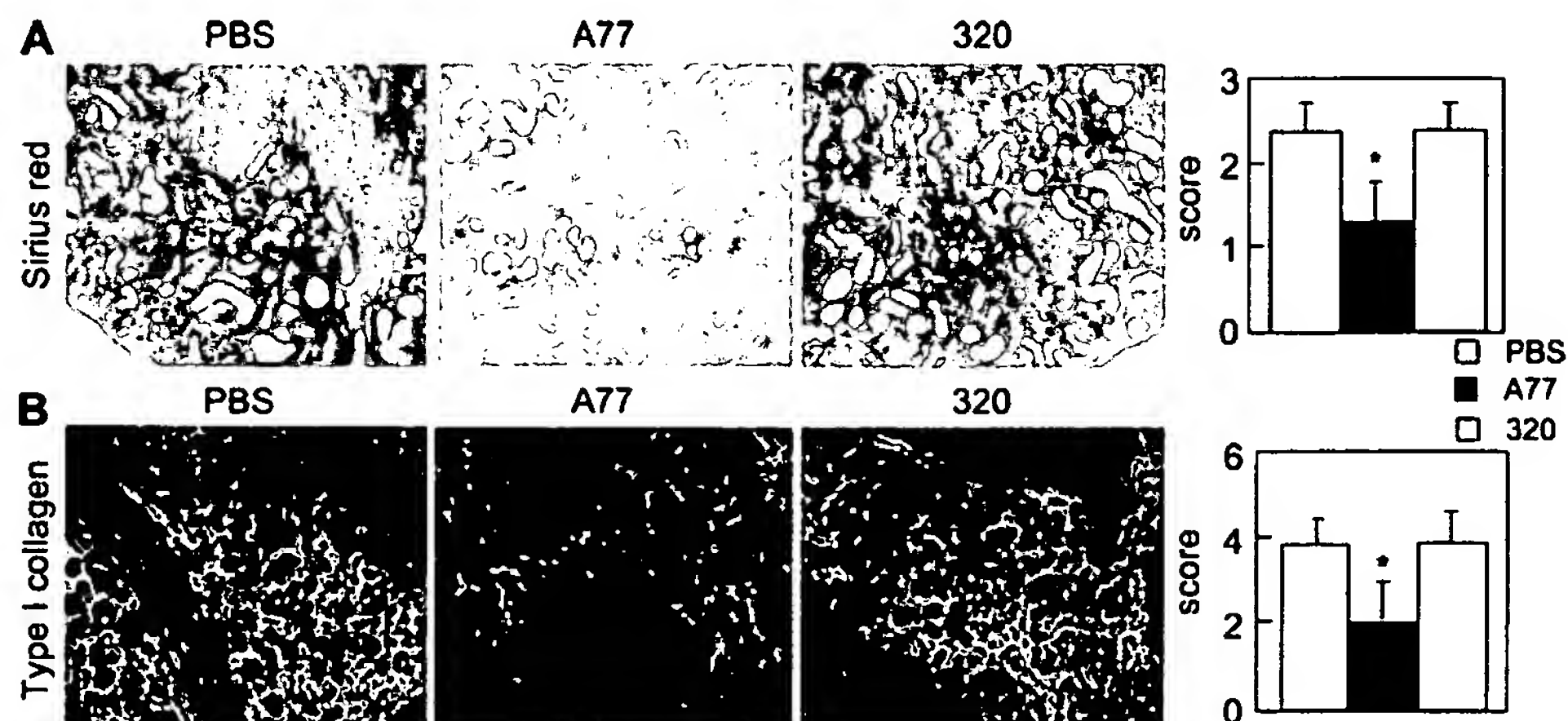


FIGURE 5. Treatment of FcαRI transgenic (Tg) mice with anti-FcαRI Fab A77 inhibits inflammatory cell infiltration after UUO. **A**, Immunohistological analysis of Mac-1-, F4/80-, and CD4-positive cells in FcαRI transgenic mice after UUO (day 14) following treatment with either PBS, Fab A77, or control Fab 320, as described in *Materials and Methods*. The corresponding quantitative analysis of infiltrating cells. *, $p < 0.05$. **B**, Immunohistological analysis of Mac-1-, F4/80-, and CD4-positive cells in FcαRI_{R209L} transgenic mice after UUO (day 14), following treatment with PBS, Fab A77, or control Fab 320. Corresponding quantitative analysis of infiltrating cells per high-power field (HPF) is shown below each staining.

We next examined the effect of Fab A77 treatment in a nonimmunological model of obstructive nephropathy induced by UUO, which involves MCP-1-induced macrophage recruitment (33–35). On day 14 after left-sided UUO in FcαRI transgenic mice, macroscopic examination showed strongly increased kidney size in control Fab 320-treated animals as compared with the nonobstructed right kidneys (data not shown). This increase was diminished in Fab A77-treated animals, indicating that inflammatory response was reduced. Further histological analysis confirmed that Fab A77-treated transgenic mice had significantly less inflammation, tubular dilatation, tubular atrophy, and mononuclear cell infiltration than control animals (Fig. 4A). Immunohistochemical

analysis showed that F4/80⁺ interstitial macrophage and CD4⁺ T cell numbers in the obstructed kidneys were lower in Fab A77-treated animals than in controls (Fig. 5A). To determine whether these effects involved active inhibitory signaling through the ITAM-containing Fcγ chain, UUO was also induced in FcαRI_{R209L} transgenic mice expressing a Fcγ-less receptor (10). A77 treatment of these mice had no effect on UUO-associated inflammation, which was similar to that in control groups (Fig. 4B). Similarly, Fab A77 had no effect on macrophage or T cell infiltration (Fig. 5B). These results confirmed our previous *in vitro* results (20) and further supported the importance of ITAM-bearing Fcγ in Fab A77-mediated inhibition.

FIGURE 6. Treatment of FcαRI transgenic (Tg) mice with anti-FcαRI Fab A77 inhibits fibrosis development after UUO. **A**, Evaluation of fibrosis in UUO kidneys (day 14) after treatment of FcαRI transgenic mice with PBS, Fab A77, or control Fab 320 as described in *Materials and Methods* using staining with Sirius red. **B**, Treated mice after immunofluorescence staining with anti-type I collagen. The corresponding quantitative evaluation of disease severity score is shown on the right. *, $p < 0.05$.



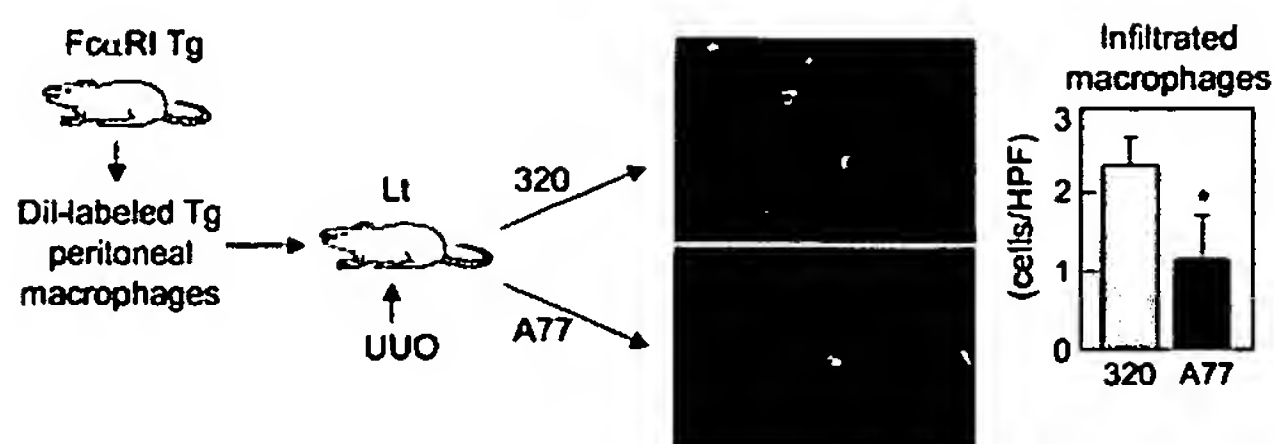


FIGURE 7. Anti-Fc α RI Fab A77 treatment directly inhibits influx of Fc α RI transgenic macrophages after UUO. Macrophages isolated from the peritoneal cavity of Fc α RI transgenic (Tg) mice were labeled with the fluorescent dye Dil and injected i.p. into Fc α RI-negative littermate (Lt) recipients 1 day before UUO. Infiltrating macrophages in UUO kidneys (day 14) were counted by fluorescence microscopy after treatment of mice with PBS, Fab A77, or control Fab 320 as described in *Materials and Methods*. A representative photograph and data obtained by analysis of 50 high-power fields (HPF) are shown. *, $p < 0.05$.

The UUO model is characterized by progressive fibrosis, fibroblast proliferation, increased accumulation of extracellular matrix proteins, and tubule atrophy (12). We therefore analyzed Sirius red-stained kidney sections (Fig. 6A) and type I collagen deposition (Fig. 6B) as markers of fibrosis in Fc α RI transgenic mice treated with Fab A77 or Fab 320. Both parameters were substantially attenuated by Fab A77 but not by Fab 320, and no such reduction was observed in Fc α RI_{R209L} transgenic mice (data not shown). Together, these results indicate that anti-Fc α RI Fab treatment has remarkable anti-inflammatory efficacy in nonimmune obstructive kidney disease.

Fc α RI monovalent targeting directly inhibits transgenic macrophage accumulation in inflamed kidney

One of the main features observed after Fab A77 treatment was the strong reduction in the inflammatory cell infiltrate in both the im-

munological and nonimmunological disease models. To determine whether this feature was due to a direct effect of Fab A77 on the homing of myeloid cells to the inflamed kidney, we examined the effect of Fab A77 treatment on macrophage recruitment in the UUO model by conducting adoptive transfer experiments. Syngenic Dil-labeled peritoneal human Fc α RI transgenic macrophages were injected i.p. into nontransgenic littermate recipients 1 day before UUO. On day 14, obstructed kidneys in mice treated with Fab 320 or Fab A77 were analyzed for the presence of fluorescent infiltrating macrophages. The number of macrophages was significantly lower in Fab A77-treated animals than in controls (Fig. 7), supporting a direct effect on macrophage homing.

Fc α RI monovalent targeting diminishes inflammatory signaling in human monocytes, independently of disease status

To analyze the therapeutic potential in humans we examined the effect of Fab A77 treatment on TNF- α production by human blood monocytes isolated from healthy subjects and patients with inflammatory and noninflammatory kidney diseases. LPS was chosen as stimulant, as it induces strong TNF- α production and because bacterial infection of the upper respiratory tract is often associated with nephropathies contributing to disease aggravation (36). As shown in Fig. 8A, unstimulated monocytes from healthy subjects, patients with lupus nephritis, and patients with antineutrophil cytoplasmic Ab-associated glomerulonephritis produced similar baseline levels of TNF- α . LPS strongly induced TNF- α secretion, to a similar degree in all groups, ruling out priming or unresponsiveness of patients' monocytes. Pretreatment with Fab A77, but not with Fab 320, led to a similar marked reduction in TNF- α production in both healthy subjects and patients. The anti-inflammatory effect of Fab A77 after LPS stimulation was confirmed in experiments showing a decrease in p38 and ERK MAPK phosphorylation in MonoMac 6 cells (Fig. 8B).

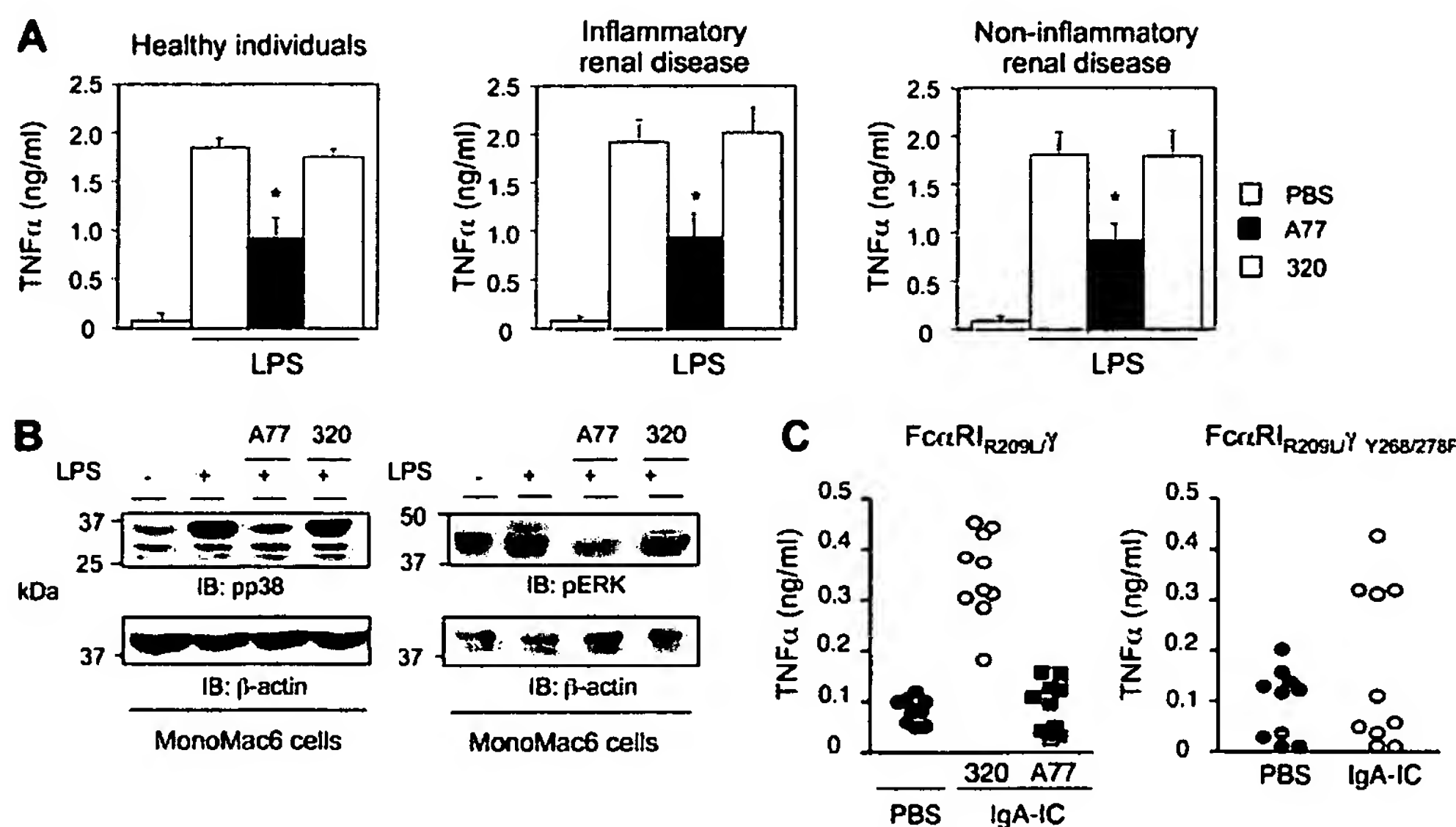


FIGURE 8. Treatment with anti-Fc α RI Fab A77 inhibits heterologous LPS-induced TNF- α production and autologous IgA immune complex-induced degranulation of Fc α RI RBL mast cell transfectants. **A**, Blood monocytes obtained from normal subjects ($n = 6$), patients with inflammatory renal disease ($n = 10$; including four antineutrophil cytoplasmic Abs (ANCA)-associated glomerulonephritis (GN) and 5 lupus nephritis), and patients with noninflammatory renal disease ($n = 10$, including four membranous nephropathy, three minimal change, and three diabetic nephropathy) were stimulated with LPS (100 ng/ml). TNF- α production was measured by ELISA and compared with unstimulated cells after preincubation (24 h) with PBS, Fab A77 (10 μ g/ml), or control Fab 320 (10 μ g/ml). *, $p < 0.05$. **B**, Representative phosphorylation response of p42–44 ERK and p38 MAPKs after LPS stimulation (100 ng/ml) of MonoMac 6 cells, as revealed by immunoblotting with the indicated phospho-specific Abs. Reprobing with anti- β -actin is shown as a control for equal loading. **C**, PBS, Fab 320 (10 μ g/ml), or Fab A77 (10 μ g/ml) treated (2 h) Fc α RI_{R209L}/FcR γ (left) and untreated ITAM-mutated Fc α RI_{R209L}/FcR γ _{Y268/278F} (right) mast cell transfectants were stimulated (3 h) with either control buffer (PBS) or PEG-purified immune complex (IC) from 10 patients (shown by each symbol) with IgA nephropathy. TNF- α release was then determined.

This extends the ITAM_i function of Fc α RI to signaling induced by TLR4.

IgA nephropathy patients have pathologic IgA immune complexes in their serum, which contribute to disease development (37). Our recent evidence points to the involvement of Fc α RI, as specific cross-linking of this receptor aggravated symptoms in a mouse model of spontaneous IgA nephropathy. Given the fact that Fab A77 binds to Fc α RI at a site different from IgA and therefore does not block IgA immune complex binding to the receptor (38, 39), we examined whether Fab A77 could inhibit responses induced by its own receptor stimulated through IgA immune complex. We used Fc α RI-humanized RBL mast cells transfected with a chimeric Fc α RI_{R209L}/Fc γ that can be activated by IgA immune complex purified by precipitation with PEG from serum of an IgA nephropathy patient (24). As shown in Fig. 8C (*left*), incubation of cells with buffer alone did not induce significant TNF- α production. Of 49 patients' sera tested, IgA immune complex from 10 patients induced significant TNF- α production when transfectants had been preincubated with control Fab 320 (Fig. 8C, *left*). In contrast, preincubation with Fab A77 strongly inhibited the TNF- α production triggered by these patients' IgA immune complex. To test the specificity of stimulation through Fc α RI, IgA immune complexes were added to transfectants expressing an ITAM-mutant chimeric receptor Fc α RI_{R209L}/Fc γ _{Y268/278F}. As shown in Fig. 8C (*right*), PEG precipitates from 6 of the 10 responder patients did not stimulate mutant receptor transfectants, suggesting that these six patients had indeed stimulatory Fc α RI-targeting IgA immune complex, whereas the other four patients appeared to contain an unidentified stimulatory factor in their precipitates. However, all responses were inhibited by A77 Fab, further supporting the broad efficacy of Fab A77 to inhibit multiple responses (Fig. 8, *left*). These results also demonstrate that monomeric targeting of Fc α RI inhibits autologous and heterologous receptor-activated responses.

Discussion

Myeloid cell-expressed Fc α RI and its associated ITAM-bearing Fc γ subunit have recently emerged as new actors in immune homeostasis (19, 20, 40). They act as a dual-function module that can either activate cells or attenuate cell activation through heterologous ITAM-bearing receptors, depending on the interaction with its ligand. Contrary to receptors bearing a bona fide inhibitory ITIM, ITAM_i-mediated inhibition does not require coaggregation of activating and inhibitory receptors. We therefore postulated that monovalent targeting of Fc α RI might not only act on other ITAM-bearing receptors but control cell activation in a more general manner. We provide evidence that induction of ITAM_i signaling through Fc α RI induces a broadly effective inhibitory signal toward responses induced by multiple inflammatory receptors and ligands. Besides inhibiting other ITAM-bearing receptors, as previously shown (20), we found that responses induced by a highly diverse set of inflammatory mediators such as MCP-1, TNF- α , and LPS were inhibited. The homologous ITAM-bearing adaptor DAP12, when associated with the isoform TREM-2, has also been found to down-regulate activating responses triggered via TLRs and Fc receptor (21, 22). Interestingly, incubation with anti-Fc α RI Fab before stimulation with IgA immune complex present in serum of IgA nephropathy patients also attenuated activation induced by its own receptor. Such autologous ITAM-dependent desensitization has also been reported to occur during T cell Ag receptor activation in response to weakly binding ligands (41). Our data support the view that monovalent targeting of Fc α RI induces a general desensitized state that interferes

with activation through a whole variety of receptors, including autologous Fc α RI.

Previous studies pointed to several distinct mechanisms responsible for ITAM-mediated inhibitory functions (reviewed in Refs. 19, 42). They included interference of PI3K and phospholipase C γ with TLR activation, intrinsic negative regulation by ITAM phosphorylation, sequestration of signaling effectors, differential vesicular targeting to intracellular signaling compartments, and production of anti-inflammatory cytokines. In the case of Fc α RI, we have proposed a role for SHP-1 phosphatase, based on preferential recruitment of SHP-1 over Syk to Fc α RI following inhibition of Fc α RI-stimulated degranulation responses. We confirm an enhanced recruitment to the phosphorylated Fc α RI-associated Fc γ chain in TNF- α -stimulated Fc α RI transgenic macrophages after incubation with Fab A77. The role of SHP-1 was directly shown by means of siRNA experiments. Specific knock down of SHP-1 markedly reduced the capacity of Fc α RI to inhibit both MCP-1- and TNF- α -induced signals identifying SHP-1 as a major factor in the Fc α RI inhibitory mechanism.

Our described broad inhibitory action of anti-Fc α RI Fab suggests its potential for a general immunotherapeutic agent for inflammatory diseases in addition to IgE-mediated asthma (20). Given its capacity to inhibit responses mediated both by immunoreceptors and by a variety of other receptors, we tested the therapeutic potential of Fc α RI targeting in immunological (anti-GBM) and nonimmunological (UUO) models of inflammatory kidney diseases. Treatment was clearly beneficial in both models. In the anti-GBM model, renal function parameters such as proteinuria, blood urea nitrogen, and creatinine levels were considerably improved by Fab A77 pretreatment, and histological signs of disease activity were attenuated. Similarly, in the UUO model, inflammatory signs were reduced, both macroscopically and histologically. Fab A77 treatment also prevented fibrosis development, as shown histologically and immunohistologically (type I collagen staining). The effectiveness of Fab A77 in UUO was abolished in Fc α RI_{R209L} transgenic mice showing that the Fc γ -less receptor is unable to mediate anti-inflammatory signaling. This establishes the role of ITAM-bearing Fc γ signaling in the Fc α RI inhibitory function in vivo.

The protective effect of Fab A77 treatment involves a major inhibition of leukocyte accumulation in the kidneys. In both disease models the number of infiltrating macrophages and T cells was strongly reduced by Fab A77 treatment. Most leukocytes found in diseased kidneys are recruited from the circulation (43, 44). In the UUO model, we showed by means of adoptive transfer experiments with Fc α RI transgenic macrophages that Fab A77 treatment directly inhibited the influx of these cells. These data indicate that Fc α RI targeting directly affects macrophage chemotaxis in vivo and are in keeping with our in vitro findings showing significantly decreased chemotaxis of THP-1 monocytic cells and purified blood monocytes in response to MCP-1 after Fab A77 treatment. As anti-inflammatory Fc α RI is mainly expressed by macrophages, our data demonstrate a deleterious role of macrophage infiltration in kidney disease and fibrosis associated with UUO. It has been reported that T cells can directly interact with renal tubular epithelial cells leading to increased production of proinflammatory proteins (45). However, as T lymphocytes do not express Fc α RI (46) our results suggest that infiltration by T lymphocytes may be secondary to activation of myeloid cells such as macrophages. Indeed, the various T cell subsets express a multitude of receptors that could respond to chemokines produced by macrophages in inflamed kidney tissue (47, 48).

We also showed the anti-inflammatory effect of monovalent targeting of Fc α RI on human monocytes ex vivo. Preincubation with Fab A77 inhibited LPS-induced TNF- α production by monocytes from healthy subjects and patients with both inflammatory and noninflammatory kidney diseases. These results are in keeping with the previously reported inhibition of FcR γ -mediated human phagocytic responses (20). They also support the notion that anti-Fc α RI Fab may be a promising treatment for human renal inflammatory diseases. Interestingly, Fab A77 also inhibited autologous responses induced by pathologic IgA immune complex present in the serum of patients with IgA nephropathy. Fc α RI activation on monocytes by IgA immune complex was recently recognized as an aggravating factor in a spontaneous experimental model of IgA nephropathy (10).

In conclusion, our findings show that monovalent targeting of myeloid-expressed Fc α RI generates a general, ITAM-dependent inhibitory signal (defined as ITAM_i) affecting responses induced by multiple inflammatory receptors and ligands. Both ITAM-dependent (49) and ITAM-independent inflammatory responses can be negatively regulated by this receptor. The inhibitory mechanism involves activation of SHP-1 likely by neutralizing receptor-activated phosphorylation responses. Fc α RI-mediated inhibition reduced inflammatory markers in both immune and nonimmune experimental models of renal disease. Likewise, ex vivo targeting of human blood monocytes led to a substantial inhibitory effect on heterologous LPS-stimulated responses and autologous responses induced by IgA immune complex. ITAM_i inhibition of myeloid cell-mediated inflammatory responses thus appears to be a promising potential treatment for renal inflammatory diseases, including IgA nephropathy.

Disclosures

The authors have no financial conflict of interest.

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